EXPLORING THE REGULATION OF MACROPINOCYTOSIS AND PHAGOCYTOSIS WHEN CELL MIGRATORY BEHAVIORS ARE CHANGED

by
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Abstracts

Cancer Metastasis is one of the most important reason that causes cancer so harmful to human body. It's been a challenge to study the mechanisms of cancer metastasis for a long time. Recently studying how macropinocytosis affects cancer formation has been a hot topic. If we can connect macropinocytosis and cell migration together, that will help to study the macropinocytosis impact on cancer metastasis. This thesis aims to explore how macropinocytosis and phagocytosis are related to cell migratory behaviors and whether they share similar genetic regulation pathways. We start with Dictyostelium cells and we changed to different cell migratory behaviors, such as fan-shape and oscillators, by lowing the threshold of signaling transduction excitable network (STEN). And then we applied macropinocytosis and phagocytosis essays to measure the macropinocytosis and phagocytosis uptake and compare between fan-shape, oscillatory cells and regular amoeboid cells. Our results indicate that macropinocytosis decreases a little when cell migratory behaviors change to fan-shape and oscillators, while phagocytosis increases a little when migratory behaviors change to fan-shape and oscillators. This can give us an idea that how macropinocytosis and phagocytosis are regulated under different migratory behaviors. Furthermore, we can relate to these cell migratory modes to cancer cells and see if there is any similarity in genetic regulation.

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Introduction

Different cells have different migratory behaviors. Even a single kind of cell can display different migratory modes at different times or under different external cues. It is important to study these transitions between migratory behaviors. Migratory transitions can occur during cancer progression, epithelial-mesenchymal transition, and immune cell synapse formation, for example. Cell migratory behaviors are regulated by many expressed genes in charge of different functions. Previous studies reported an excitable signaling transduction network (STEN) which is involved in directed cell migration [1]. Signal transduction components involved in this network are connected together by multiple parallel or complementary pathways, including phosphoinositides and Ras superfamily GTPases (Figure 1) [1]. These linkages lead to biochemical excitability manifested as a refractory period and all-or-nothing responses. Previous experiments done in our lab proved that lowering phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) or increasing Ras/Rap GTPases will activate Ras activity. cAMP serves as external cue to STEN and it contributes to activation of Ras. Therefore, lowering PIP2 or increasing Ras/Rap will decrease the cAMP level required for a given response. That is, the threshold of STEN is lowered. This system has the advantage that small changes can be greatly amplified. We can change cell activities at any position and time point. Thermal fluctuations amplify stochastic noises to the network, resulting in getting protrusions, wave propagations, etc. Since this network is excitable, perturbations at crucial nodes in the network will display striking phenotypes (Figure 2) [9]. For example, if we lower the threshold of STEN, cells will display fan-shaped and oscillatory behaviors.

If we increase STEN threshold, cells will move with spiky filopods. If we inhibit STEN, there's ruffles protrusions everywhere and cells will be immobilized.

A question to be learned is that once cell migratory behaviors are changed, whether other cell activities will be affected. Among all kinds of cell activities, I chose to study macropinocytosis and phagocytosis first since the ability of capturing and engulfing extracellular materials is one of the important cellular functions [3]. Macropinocytosis is an endocytic process whereby cells engulf extracellular fluid as a nutrient source from the environment. Protrusions of plasma membrane, driven by actin polymerization and myosin contraction, form cup-shaped circular ruffles with a diameter of several microns. The ruffle engulfs and transfers extracellular material through cell membrane in a structure called a macropinosome. Macropinosomes travel through the endocytosis system and their large contents can be decomposed by digestive enzymes and useful metabolites extracted [4]. Phagocytosis is another endocytic process whereby living cells called phagocytes engulf large foreign solid particles and bacteria. Amoebae are a kind of phagocyte. Phagocytosis recognizes and binds extracellular materials by receptors on the cell surface. Some professional phagocytes such as dendritic cells, neutrophils, and macrophages, can clear the infection sites that invaded by foreign substances, like bacteria and fungi. Phagocytosis is not only important for the first line of clearing infection, but also plays an important role in antigen presentation [5]. For example, dendric cells engulf pathogens and present antigen to T cells. *Dictyostelium* cells has been developed as the simplest model to study the molecular mechanisms regulating macropinocytosis and phagocytosis since it can engulf particles and fluids [6]. *Dictyostelium* cells also allow us to observe cell motility and endocytosis process together.

As we study more about the mechanisms to generate macropinocytosis and phagocytosis, it is becoming clear that there is some similarities in signaling pathways that regulate protrusions formation that drive cell migration [10]. Figure 3 [10] shows the relationship between endocytosis and cell migration. Therefore, we hypothesize that signaling pathways involved in regulating cell migration will also regulate macropinocytosis and phagocytosis activity, and furthermore, that both are affected by the threshold of this excitable signaling transduction network. By perturbating some important signaling components involved in STEN, such as PI(4,5)P2 and Ras/Rap GTPases, the threshold of STEN can be lowered. I have been able to observe the change of cell migratory behaviors and endocytosis activities in *Dictyostelium* cells. The reason that cancer cells so harmful is because they have a much better ability to engulf more nutrients from extracellular materials. If we can observe much higher level endocytosis activities in one kind of cell migratory mode in *Dictyostelium* cells, we can relate this migratory mode to cancer cells and further study if they have any other similarities in genetic regulation.

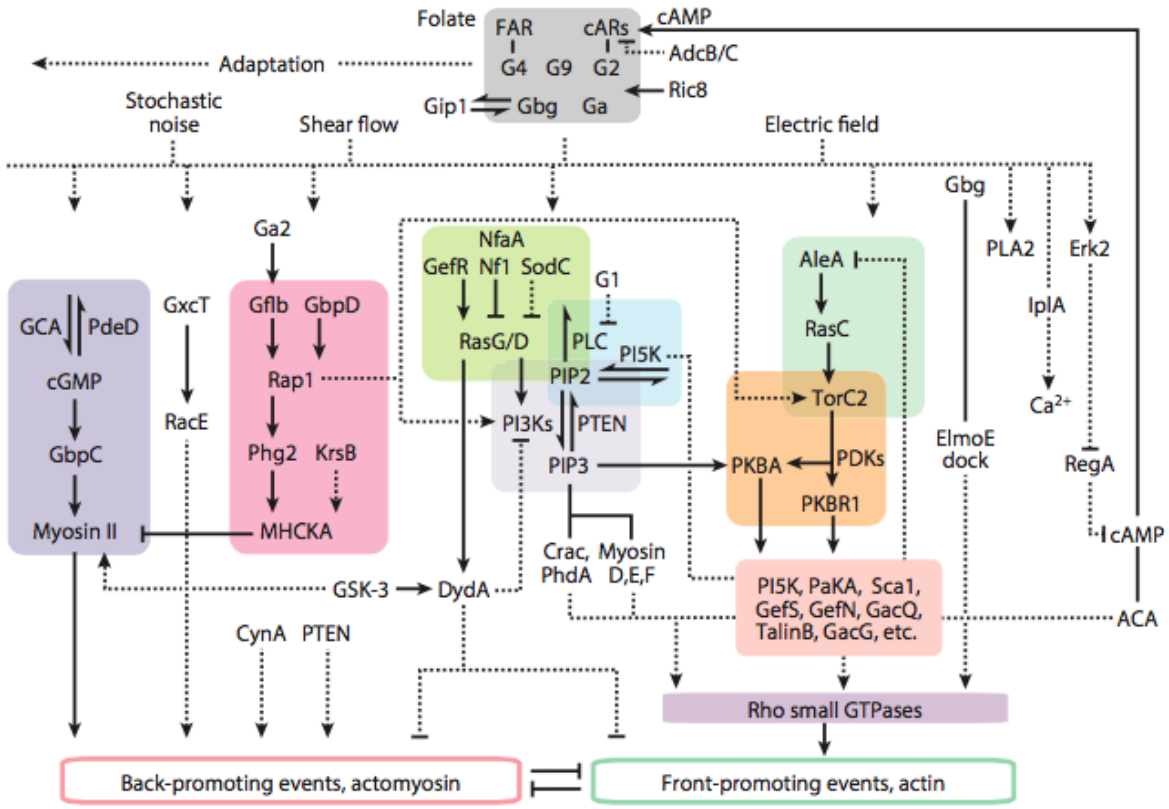


Figure 1 The network of signal transduction pathways involved in directed cell migration in Dictyostelium.

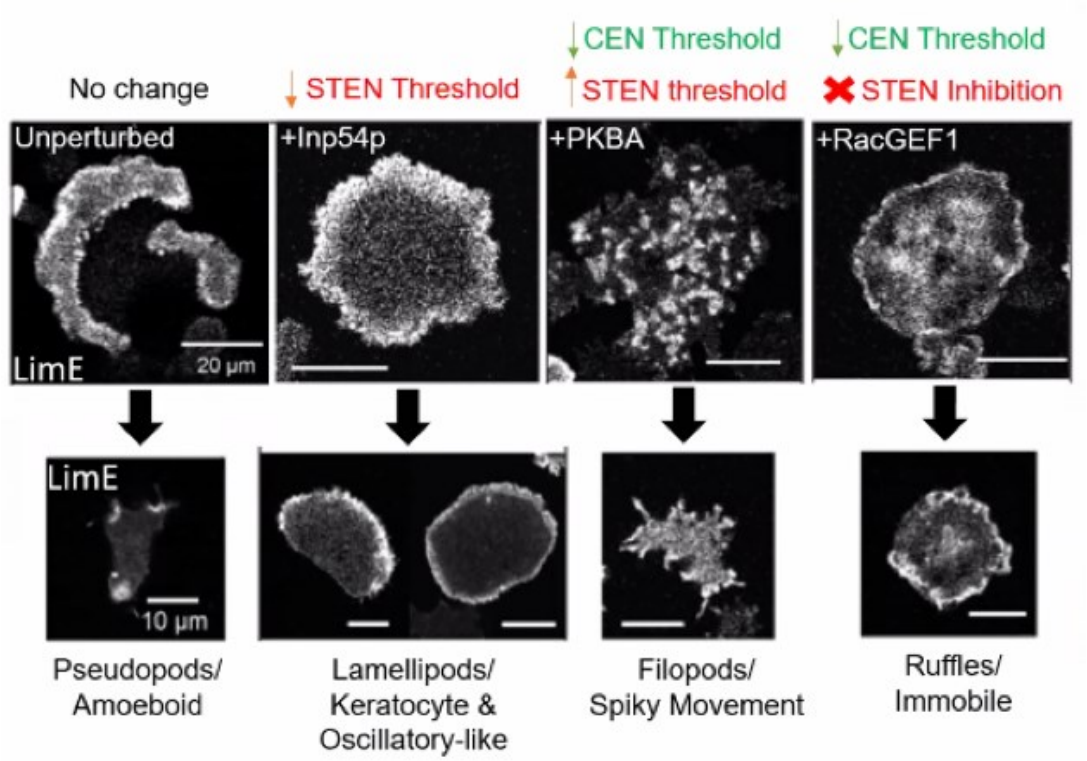


Figure 2 Confocal image showing different wave patterns (top) and cellular protrusions (bottom) under different perturbations. From left to right: 1) No threshold change of STEN/CEN; 2) Lowering the threshold of STEN by lowering PI(4,5)P2 with recruitment of Inp54p; 3) Increasing the threshold of STEN and lowering the threshold of CEN by recruitment of; 4) Inhabiting STEN and loweing the threshold of CEN by recruitment of RacGEF1.

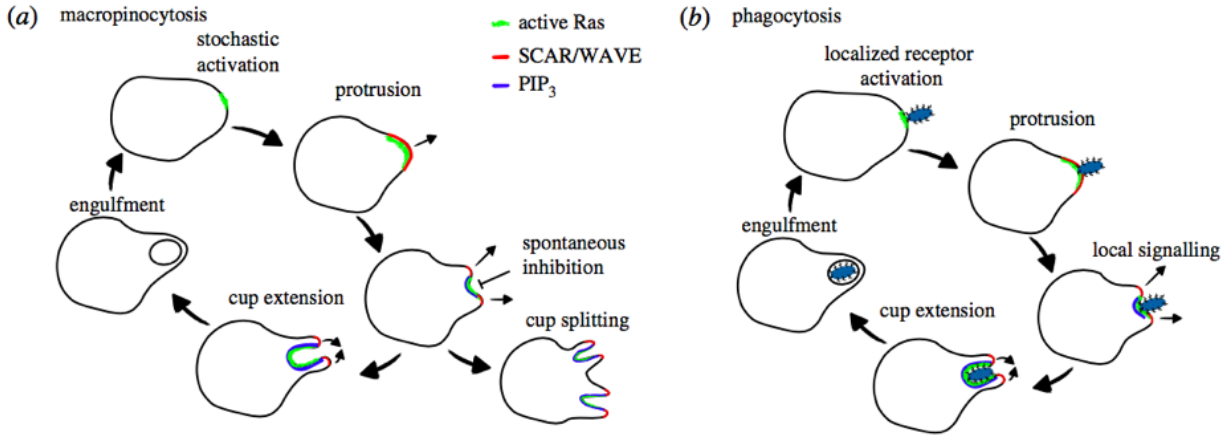


Figure 3 the relationship between endocytosis and cell migration. A shared mechanism is used to initiate cell migration and generate the cup-shaped protrusions required for macropinocytosis and phagocytosis

Results

Recreate Fan-shape and oscillatory cells by lowering PI(4,5)P2 or increasing Ras/Rap GTPases levels.

To recreate fan-shape and oscillatory cells, I followed the method of a former graduate student using a chemically inducible dimerization (CID) system in *Dictyostelium* to lower PI(4,5)P2 levels or increase Ras/Rap GTPase levels. Figure 4 shows the mechanism of the CID system [2].

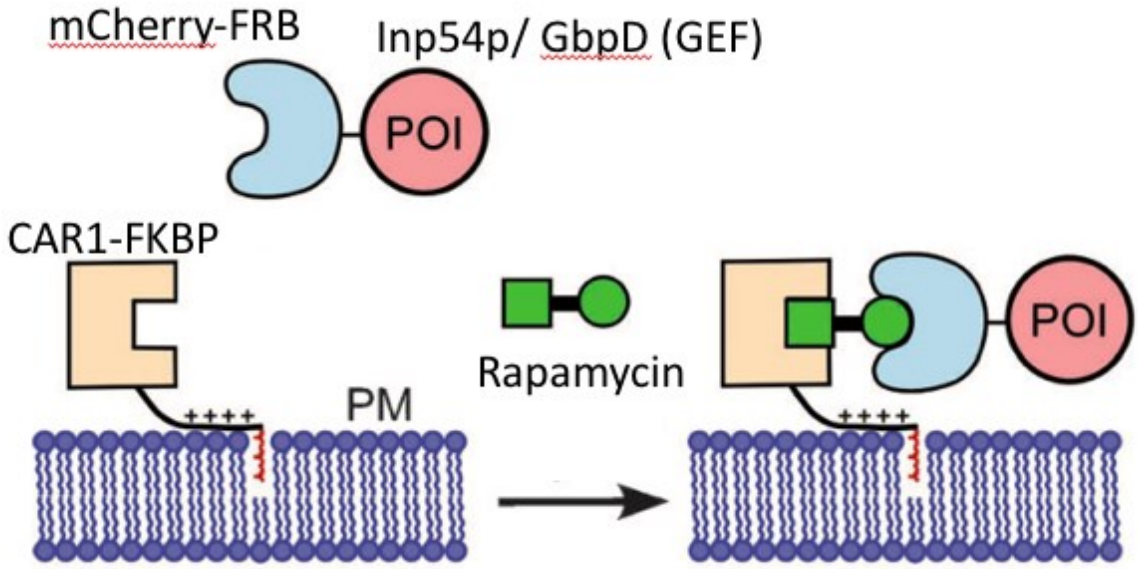


Figure 4 Scheme of CID system. FKBP and FRB are proteins that can bind together only when small molecules or drugs (Rapamycin) is present. Upon adding Rapamycin, proteins of interest that attached to FRB can be recruited to where FKBP protein located.

cAR1-FKBP-FKBP and mCherry-FRB-Inp54p were co-expressed. cAR1 is a membrane targeting protein and cAR1-FKBP-FKBP was observed to be almost exclusively on the membrane. FRB can be attached any protein of interest to be recruited. Upon addition of Rapamycin, FRB and FKBP bind and consequently Inp54p, for example, can be rapidly and irreversibly recruited to plasma membrane. Through this recruitment, PI(4,5)P2 levels were reported to decrease about 90% and the threshold of this signaling transduction network is decreased. In my hands, after about 30 mins of recruitment, a fraction of the amoeboid cells switched to fan-shape or oscillatory behaviors (Figure 6). "Fan-shape" cells are similar to gliding keratocytes, with wide protrusions along the anterior, and with a flat and contractile back. “Oscillators” have a repeating cycle of spreading globally first, then contracting strongly, then spreading again. Between each cycle, cells will migrate briefly. Both fan-shape and oscillators display much faster migrating speed and longer distance compared with amoeboid cells (Figure 5). Velocity (μm/min) for fan-shape cells

is about 13.8 µm/min and amoeboid cells is about 6.8 µm/min. There are maximum 20% fan-shape and 50% oscillators among the cell population after recruitment [2].

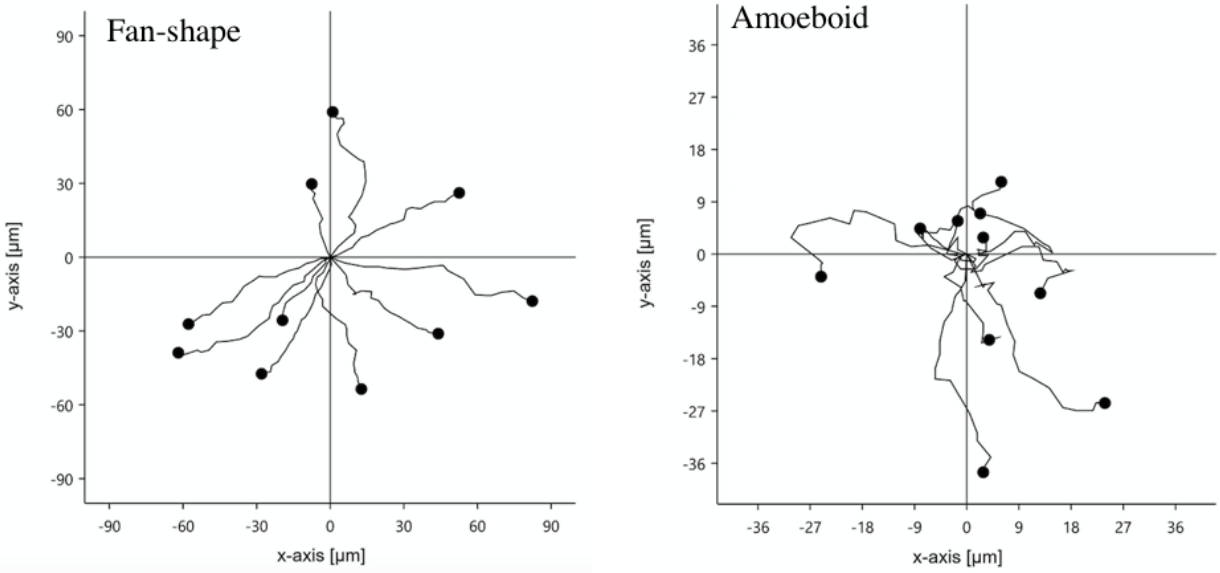


Figure 5 Cell movement trajectory of fan-shape cells (left) and amoeboid cells (right). Each track lasts 5 min. Velocity (µm/min) is 6.8 for amoeboid, 13.8 for fan-shaped.

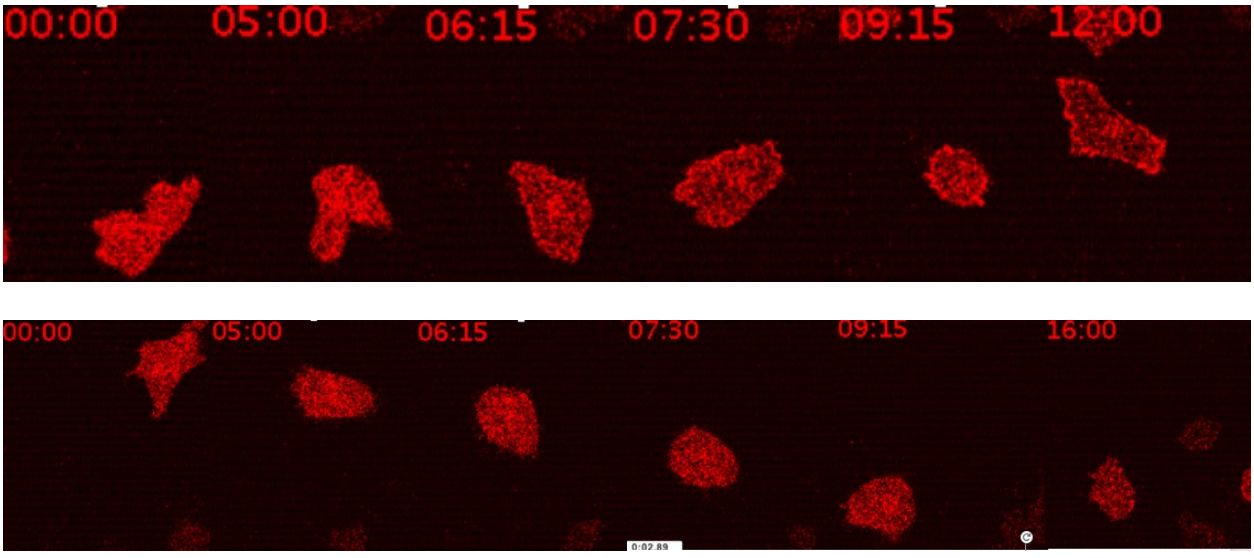
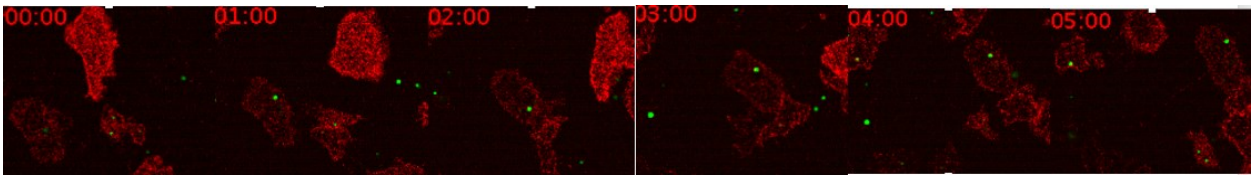


Figure 6 Time-lapse phase-contrast images showing the transition of an amoeboid cell to an oscillatory (top) or a fan-shape cell (bottom) under Inp54p recruitment. Scale bars in all images represent 10 µm.

Macropinocytosis uptake decreases when the threshold of STEN is lowered

After successfully generating fan-shape and oscillatory cells, I performed macropinocytosis assays on either fan-shape/oscillators or Inp54p cells without recruitment to test the relation between macropinocytosis uptake and different cell migratory modes. Figure 7 showed the macropinocytosis uptake for Inp54p recruited cells (top) and Inp54p cells without recruitment (bottom). When I quantified FITC intensity for macropinocytosis uptake of fan/oscillator cells, I selected cells which display fan/oscillators patterns more than 5 mins in the movies as our defined "fan/oscillator cells". After collecting data of about 300 cells for each type, I summarized the FITC intensity difference between fan-shape and WT cells with a histogram (Figure 8). There are 50% fan-shape cells with a FITC intensity that lowers than 400, while there are 46% non-recruited cells in this range. In the high FITC intensity range which intensity is over 700, there are 19.7% fan-shape cells and 26.7% non-recruited cells. As shown in the graph, there is a slight shifting of the peak to lowered intensity range for fan-shape cells upon Inp54p recruitment compared with Inp54p cells without recruitment under same FITC-dextran incubation time. This suggests that macropinocytosis uptake may decrease when cell migratory mode change from amoeba-like to fan-shape/oscillatory behaviors by lowering PIP2 activities. Unfortunately, I was not able to collect sufficient data to conclude that the difference is significant.



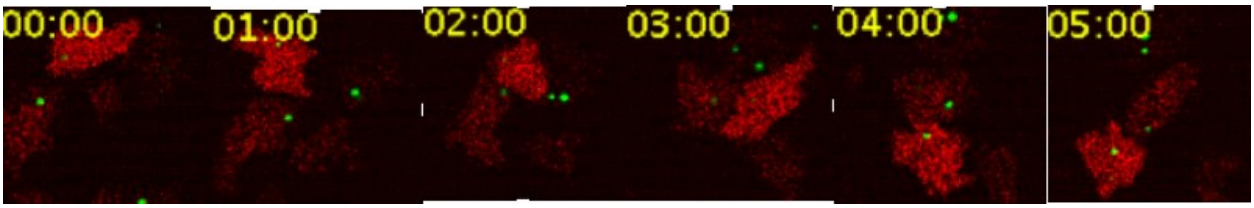


Figure 7 Macropinocytosis uptake with FITC-dextran of Fan-shape cells (top) under Inp54p recruitment and Inp54p cells without recruitment (Bottom). Scale bars in all images represent 10 µm.

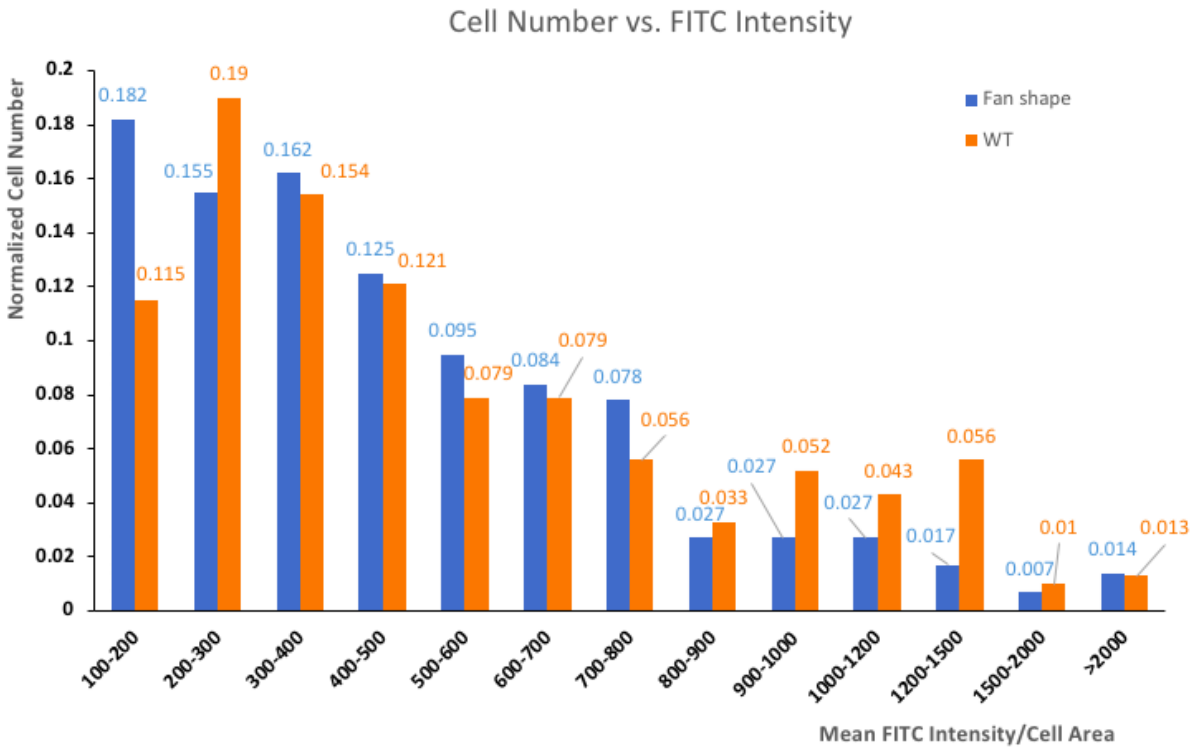


Figure 8 FITC intensity difference between fan-shape cells upon Inp54p recruitment and Inp54p cells without recruitment. y-axis is normalized cell number and x-axis is mean FITC Intensity per cell area.

I also plan to compare macropinocytosis uptake between induction with doxycycline of constitutively active $RasC_{Q62L}$ versus $RasC_{WT}$. Previous experiments done in our lab proved that induction of the constitutively active $RasC_{Q62L}$ or $Rap1_{G12V}$ cause cells spread moderately and cells displayed oscillatory behaviors, repeating a cycle of expanding and contracting [11]. Therefore, I can induce the constitutively active $RasC_{Q62L}$ with doxycycline to get oscillatory cells and apply

macropinocytosis assay on them. This will also help me understand how macropinocytosis uptake changes when Ras activity level is increased in the network. Figure 9 showed macropinocytosis uptake with FITC-dextran of constitutively active $RasC_{Q62L}$ (top) under doxycycline induction and $RasC_{WT}$ (Bottom). I will continue to get more data and finish the quantification.

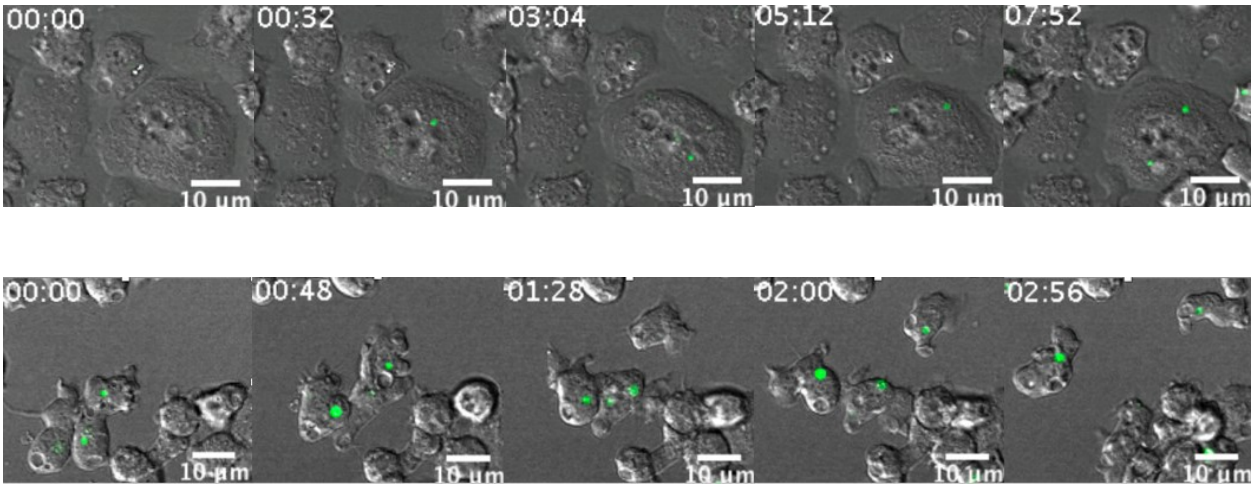


Figure 9 Macropinocytosis uptake with FITC-dextran of constitutively active $RasC_{Q62L}$ (top) under doxycycline induction and $RasC_{WT}$ (Bottom). Scale bars in all images represent 10 µm.

Phagocytosis uptake increases when the threshold of STEN is lowered

To verify that my phagocytosis assay is effective, I first applied the assay using non-fluorescence labeled-yeast cells on LimE-GFP expressing cells. Figure 10 shows the phagocytosis engulfment process in LimE-GFP expressing cells. We can observe the phagocytic cup clearly and the whole process of cells engulfing yeast. This experiment indicated that the phagocytosis assay is effective.

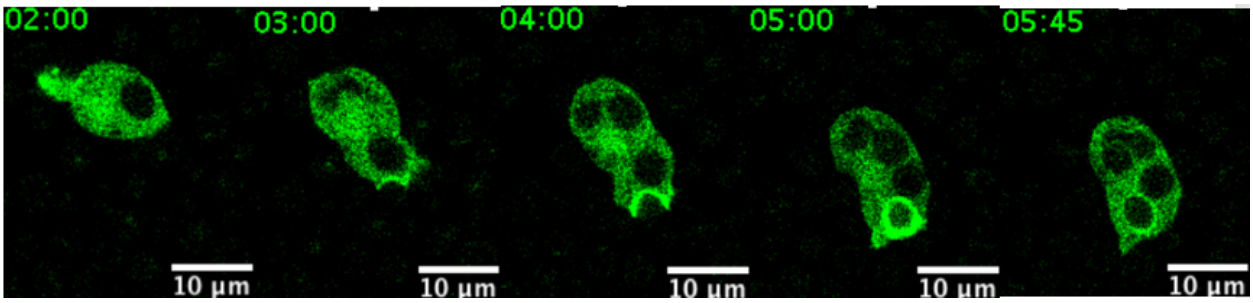


Figure 10 Phagocytosis process of Lime-GFP expressed cells by engulfing yeast cells. Scale bars in all images represent 10 µm.

Next I performed this phagocytosis assay on Inp54p recruited cells and Inp54p cells without recruitment. Figure 11 shows phagocytosis engulfment of Inp54p cells with recruitment (top) and without recruitment (bottom). After collecting data of about 500 cells for each type, I summarized the engulfed yeast number difference between fan shape cells versus Inp54p cells without recruitment (left) and Inp54p recruited cells versus Inp54p cells without recruitment (right) with histogram graphs (Figure 12). There are 94.3% fan-shape cells with a yeast number that lowers than 2, while there are 95.7% WT cells in this range. In the high yeast number range which yeast number is at least 3, there are 5.8% fan-shape cells and 4.2% WT cells. In the experiments between recruited and non-recruited cells, there are only 87.7% Inp54p recruited cells with a yeast number that lowers than 2, while there are 95.7% WT cells in this range. In the high yeast number range which yeast number is at least 3, there are 12.2% Inp54p recruited cells and 4.2% WT cells. Combining results, it seems like phagocytosis uptake may increase slightly when the migratory mode changes from amoeba-like to fan-shape/oscillatory behaviors by lowering PIP2 activities. More data will need to be collected to reach a significant conclusion.

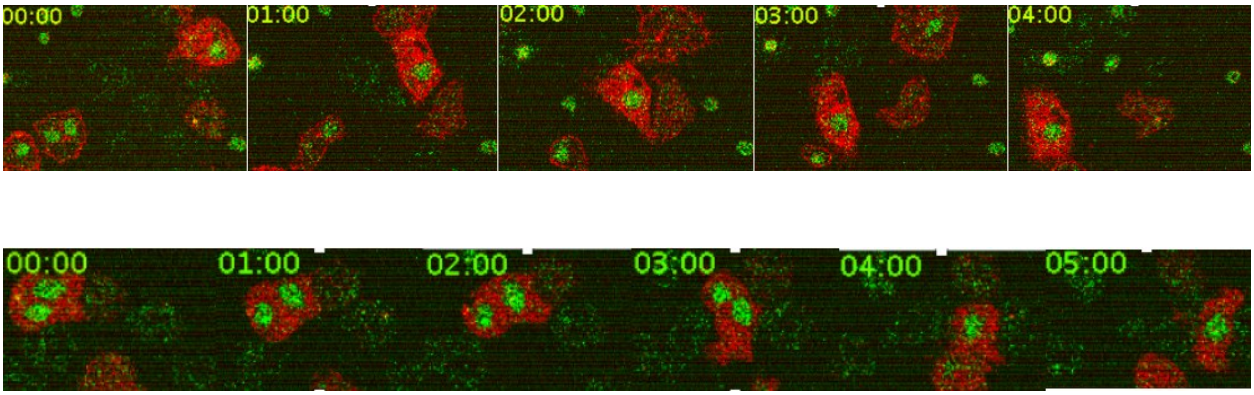


Figure 11 phagocytosis engulfment of Inp54p cells with recruitment (fan-shape) (top) and without recruitment (bottom). Scale bars in all images represent 10 µm.

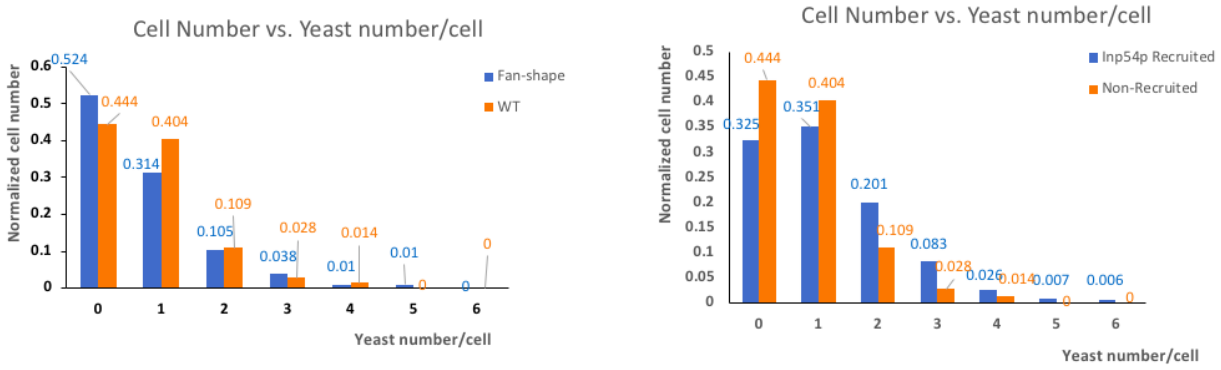


Figure 12 Yeast uptake difference between fan-shape cells upon Inp54p recruitment and Inp54p cells without recruitment. y-axis is normalized cell number and x-axis is maximum yeast number per cell.

The same phagocytosis assay was performed on recruited cyclic GMP-binding protein D (Guanine nucleotide exchange factors) GbpD(GEF) cells with or without recruitment and I obtained similar results compared with Inp54p recruitment. There is a peak shifting to higher engulfed yeast number range for fan/oscillators compared with WT upon GbpD(GEF) recruitment (Figure 13). There are 67.2% oscillator cells with a yeast number that lowers than 3, while there are 75.4% WT cells in this range. In the high yeast number range which yeast number is at least 3, there are 32.7% oscillator cells and 24.5% WT cells. In the experiments between recruited and

non-recruited cells, there are 70.3% GbpD(GEF) recruited cells with a yeast number that lowers than 3, while there are 75.4% WT cells in this range. In the high yeast number range which yeast number is at least 3, there are 29.7% GbpD(GEF) recruited cells and 24.5% WT cells. Combining results, it seems like phagocytosis uptake may increase slightly when the migratory mode changes from amoeba-like to fan-shape/oscillatory behaviors by increasing Ras/Rap activities.

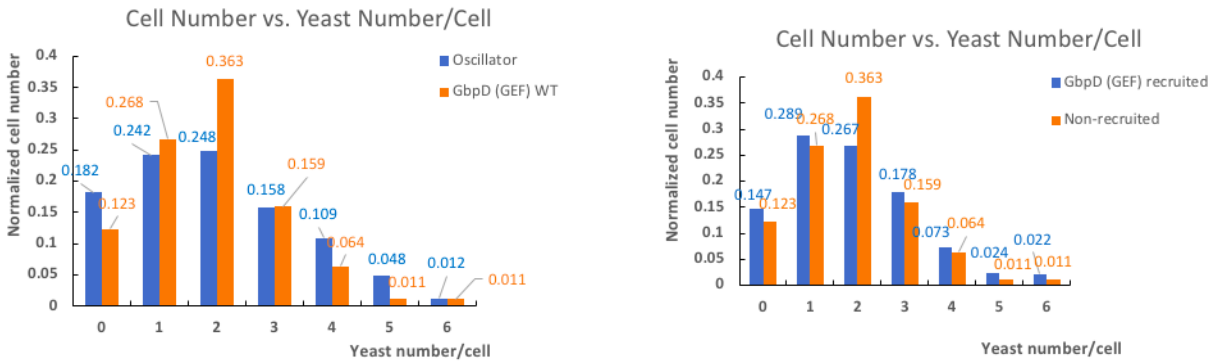


Figure 13 Yeast uptake difference between oscillatory cells upon GbpD (GEF) recruitment and GbpD (GEF) cells without recruitment. y-axis is normalized cell number and x-axis is maximum yeast number per cell.

Design a T2A self-cleavage tool to increase recruitment efficiency

To increase the expression level of CID and optogenetic components, I also designed a T2A self-cleavage tool. T2A is a 20 aa-long peptide, which can induce ribosomal skipping during translation of a protein in a cell. T2A is widely used to cleave a longer peptide into two shorter peptides. Figure 14 showed the mechanism of 2A peptide cleavage. The apparent cleavage is triggered by ribosomal skipping of the peptide bond between Proline and Glycine in C-terminal of T2A peptide, resulting in the peptide located upstream of the T2A peptide to have an extra amino acid on its C-terminal end while the peptide located downstream of the T2A peptide have an extra amino acid on its N-terminal end. N150 is a targeting peptide that directs the transport

of a protein to plasma membrane of the cell. N150-GFP will label the membrane green. I designed my T2A system start with pDM358-N150-GFP. T2A piece was first cloned into this plasmid to get pDM358-N150-GFP-T2A. Then mCherry was inserted after T2A to get pDM358-N150-GFP-T2A-mCherry. This plasmid can help to differentiate whether T2A self-cleaved successfully under confocal microscopy. If T2A cleaves, then the cell membrane will be green only, and mCherry will be in the cytosol only. If T2A cannot cleave, both GFP and mCherry will be on the membrane and the membrane will display yellow (Figure 14). Another advantage of this tool is that there are restriction enzyme sites before and after GFP and mCherry sequence. GFP and Cherry can be easily replaced to other proteins and widely applied to any cells which need 2 plasmids co-expression, making the drug selection process easier and cells growing faster.

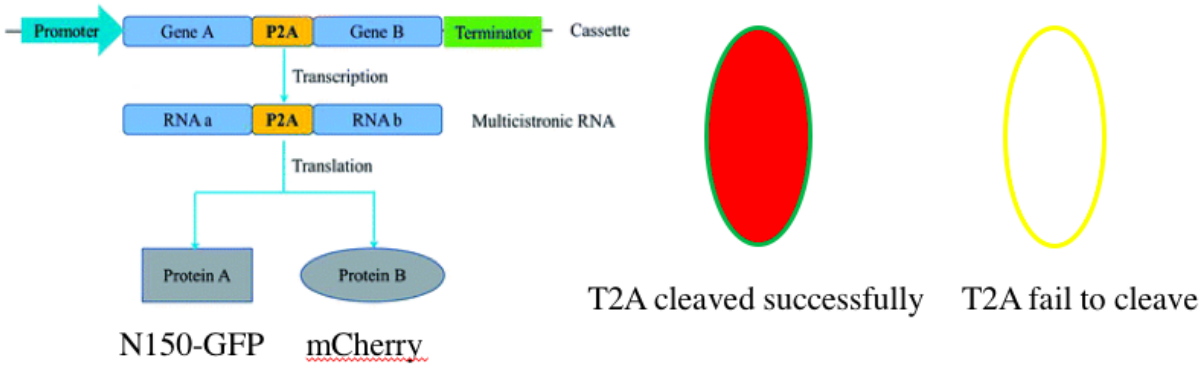


Figure 14 Scheme of T2A self-cleavage system

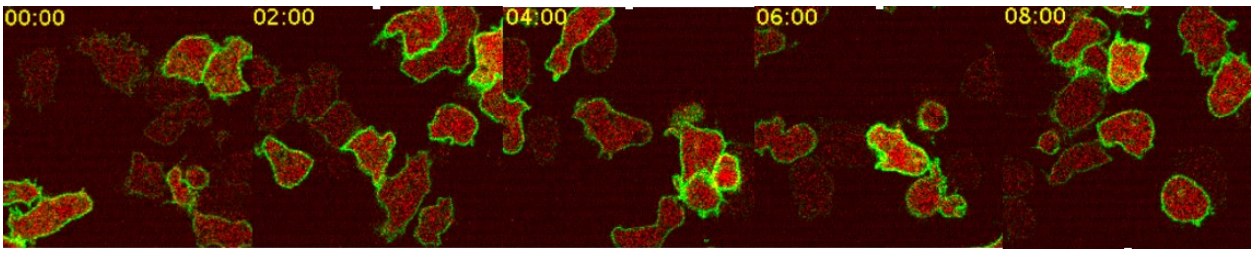


Figure 15 Time-lapse phase-contrast image showing T2A cleaved successfully

N150-GFP-T2A-mCherry was then transfected into cells to observe how cells express under confocal microscopy. Figure 15 showed that T2A cleaved successfully and cells have a very high expression level. Majority cells express GFP on membrane only while mCherry is found in the cytosol only. To further understand how many portion cells expressing T2A well and how many cells fail to cleave, I performed western blotting. Figure 16 showed the WB results.

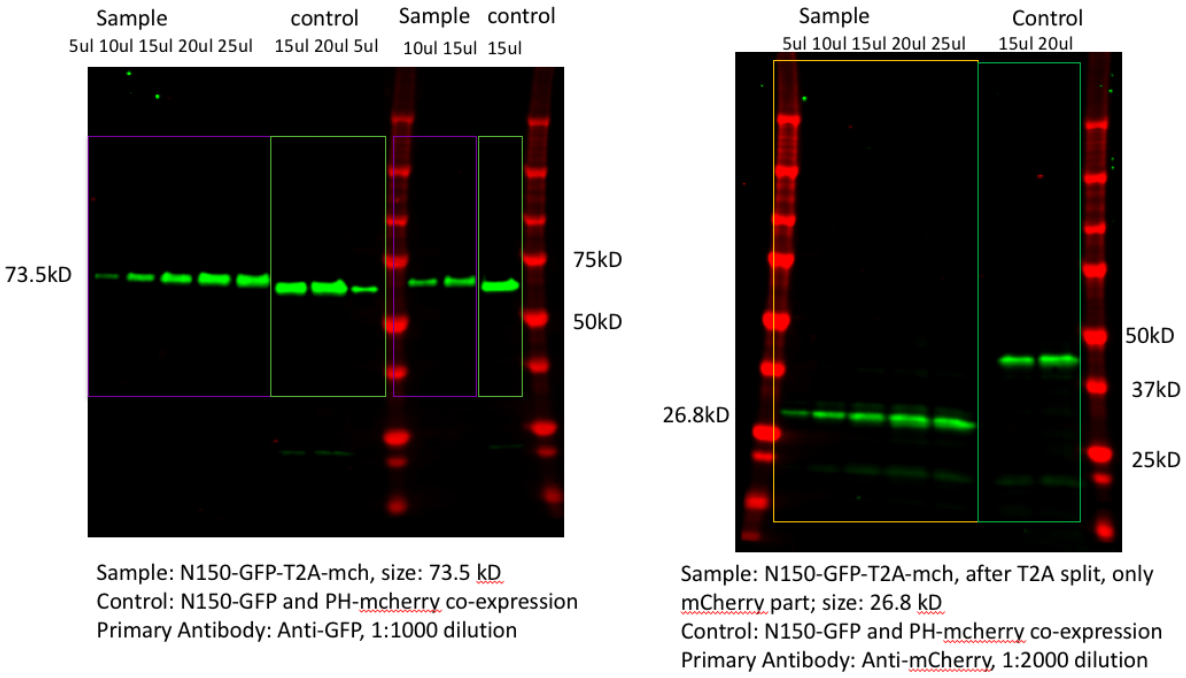


Figure 16 WB result of N150-GFP-T2A-mCherry and its control. (Left) is Anti-GFP result, (Right) is Anti-mCherry result.

From the WB results, we can tell that T2A cleaved very well, the mCherry band and GFP band are clean and we cannot detect the N150-GFP-T2A-mCherry band according to MW. There's a problem in that the N150-GFP control MW is larger than the result calculated according to sequence result. Next another control N150-mCherry was tested to check if N150 is actually bigger than the sequence result. It was consistent, ruling out an anomalous effect of the label.

We do not understand why N150 runs too large on the gel but, nevertheless we can conclude that the TA2 tool worked extremely well.

In the future I plan to apply this tool on our CID recruitment system to further test whether T2A self-cleavage can help increase the recruitment efficiency. GFP will be replaced to CAR1-FKBP-FKBP and mCherry will be replaced to mCherry-FRB-Inp54p. Then pDM358-CAR1-FKBP-FKBP-T2A-mCherry-FRB-Inp54p will be transfected into cells and imaged under confocal microscopy to test whether T2A can split well and improve recruitment efficiency.

Discussion

Previous studies reported that as STEN activities increases, cells will display different migratory mode, gradually switching from amoeboid random migration to fan-shape and oscillators (Figure 17) [1]. As STEN activity increase, the cup-shaped circular ruffles formed by protrusion extension are bigger and bigger. We usually think larger macropinocytoic cups will engulf more extracellular materials. The phagocytosis assay results match this theory. Both Oscillator cells and fan-shape cells increased cell engulf yeast ability by a bit compared with non-recruited cells. This proved that when cells form larger phagocytic cups, cells potentially can engulf more nutrients from external materials. Cancer cells also have a better ability to engulf both liquid and particulate nutrients. Since we observed a higher evel phagocytosis activity in fan-shape and oscillatory cells in *Dictyostelium*, we may be able to relate these migratory behaviors to cancer cells and further study if they share any signaling pathways in regulating cancer metastasis.

Surprisingly, macropinocytosis and phagocytosis assay results did not match. The difference of macropinocytosis uptake between fan-shape and WT cells is not significant. One of

the reasons could affect results is the FITC-dextran incubation time. We think the macropinocytosis uptake rate among fan-shape, oscillatory, and non-recruited cells may be more different at shorter times. However, if the dextran incubation is long enough, the uptake becomes saturated and eventually will be same among fan-shape, oscillatory, and non-recruited cells. Our incubation time is 15 min and this may be a relatively long time. Next we plan to perform macropinocytosis assay in shorter time (8 min, 5min, 4min, 3min, 2min, 30s) to see whether time can affect the macropinocytosis uptake amount. If we proved time plays a role in affecting results, next we will adjust our dextran incubating time to the correct time and use the same assay to acquire new data. Another reason to explain this result is that the macropinocytic cups of fan-shape cells are too big to form circular ruffles, making the engulfment ability decrease. We can do another experiment to study how macropinosomes access into fan-shape cells to get a picture of how fan-shape cells engulf particles. To get more precise statistical result, I will use unpaired two-tailed t test to get p value. A P value of <0.05 can be considered significant.

To sum up, we still do not know why the macropinocytosis and phagocytosis activities did not match when STEN activity increases. We need more statistical analysis and we need to apply assays on cells which are regulated by other signaling components to further study macropinocytosis and phagocytosis activities.

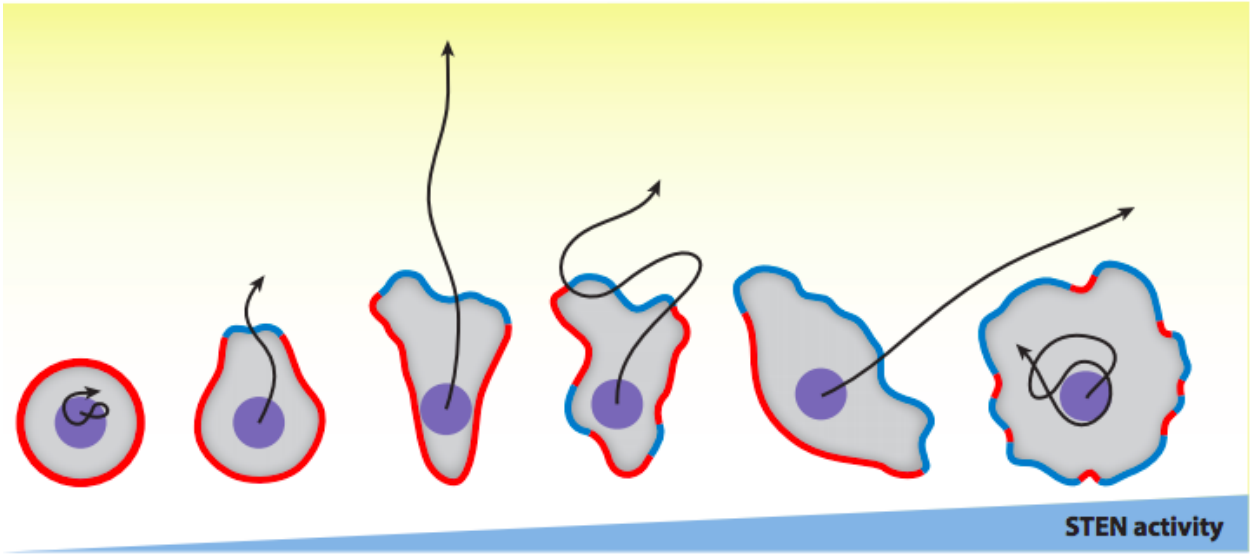


Figure 17 Different Cell migratory modes with increasing STEN activity.

Materials and Methods

Plasmids

The constructs expressing *Dictyostelium* cAR1-FKBP-FKBP, limE-GFP, N150-GFP, N150-GFP-T2A-mCherry were cloned in the vector pDM358. The constructs expressing mCherry-FRB-inp54p, mCherry-FRB-GbpD (GEF) were cloned in the vector pCV5. The construct expressing PHcarc-mCherry is cloned in the vector pDM181. The construct expressing constitutively active $RasC_{Q62L}$ is cloned in the vector pDM359.

Cell line and Cell culture

For all experiments, wild-type *Dictyostelium discoideum* of the AX2 strains were obtained from the R. Kaylaboratory (MRC Laboratory of Molecular Biology, UK) and grown in HL5 medium on tissue culture treated plastic 100 mm (Greiner CELLSTAR) or 150 mm (Falcon) plates to 70 to 90% confluency at 22 °C. Fluorescent imaging was performed in developmental buffer (DB) [phosphate buffer (PB) supplemented with 2 mM MgSO4 and 0.2 mM CaCl2]. For plasmid

transformation, cells were transformed using electroporati. Cells carrying expression constructs were placed under antibiotic selection using either 20 to 30 ug/mL G418 or 50 ug/mL Hygromycin B.

Macropinocytosis Assay

Two sets of cells were seeded in 8-well chambered coverglass (Thermo Scientific™,155409) in HL5 medium for 15min and then changed to developmental buffer (DB): 1)Inp54p cells were recruited for 30 mins and 2)Inp54p cells without recruitment. After that, FITC labeled dextran (70k MW, Sigma-Aldrich, 46945) was added to a final concentration of 2 mg/ml and incubated for 15mins at 22 ^{0}C (dissolve the powder in ddH2O to a concentration of 50 mg/ml for storage at 4 ^{0}C). FITC-dextran can load into macropinosomes in cells and display green fluorescence at 488 nm excitation/emission 521 nm. We can observe macropinosomes within cells and measure the fluorescence intensity with the Ziess 780 Confocal Microscope. After incubation, both sets cells were washed with DB three times and stored in 500 ul DB for imaging with Zesis 780 Confocal Microscopy. For each image, macropinocytosis was analyzed by outlining each cell in the images and measuring the fluorescence intensity within the outline divided by the cell area. 3-4 frames within each movie at different time point were picked to record the intensity of each cell and calculated the average to get the final mean FITC intensity. The same method was used to get the mean FITC intensity for Inp54p cells without recruitment.

Preparation of Non-Fluorescent Yeast Cells

To prepare heat-killed yeast [8], first 5g of yeast (YSC-2, Sigma) was suspended in 50ml PBS in a 100-ml Erlenmeyer flask. Maintain for 30 mins in a boiling water bath and stirred. Then yeast

cells were harvested by centrifugation at 3000g for 5 mins. Yeast pellet was washed 5 times with PBS and twice with DB. Finally, the suspension concentration was adjusted to $1*10^9$ particles /ml in DB and stored at -20 ^{0}C in 1ml aliquots.

Preparation of Fluorescent Yeast Cells

To make FITC labeled-yeast cells [8], 20ml unlabeled yeast suspension ($1*10^9$ particles /ml in DB) was centrifuged at 3000g for 5 mins. The pellet was resuspended in 20ml labeling buffer (50 mM Na_2HPO_4, pH 9.2. Add FITC dextran to 0.1 mg/ml final concentration) and incubated at 37 ^{0}C on a rotary shaker (250 rpm) for 30mins. Then the suspension was centrifuged at 3000g for 5 mins. The pellet was washed twice with labeling buffer lacking FITC dextran and 4 times with DB. Finally, the suspension concentration was adjusted to $1*10^9$ particles /ml in DB and stored at -20 ^{0}C in 1ml aliquots protected from the light.

Phagocytosis Assay

Lime-GFP expressed cells were seeded in 8-well chambered coverglass (Thermo Scientific™,155409) in HL5 medium for 15mins and then changed to DB. After 10 mins, yeast suspension was added to coverglass and mixed well with DB to make yeast to cell ratio 1:1. After 5-10 mins, both cells and yeast were adhered well on chamber surface and cells are ready for imaging with Zesis 780 Confocal Microscopy.

Two sets of cells were seeded in 8-well chambered coverglass (Thermo Scientific™,155409) in HL5 medium for 15min and then changed to developmental buffer (DB): 1)Inp54p cells were recruited for 30 mins and 2)Inp54p cells without recruitment. After that, FITC labeled-yeast cell

suspension is added to coverglass and mixed well with DB to make yeast to cell ratio is 1:1. After 5-10 mins, both cells and yeast were adhered well on chamber surface and cells are ready for imaging with Zesis 780 Confocal Microscopy. For each image, phagocytosis was analyzed by counting the yeast particles within each cell. 3-4 frames within each movie at different time point were picked to record the yeast number of each cell and the maximum number was used as the final yeast number that each cell engulfs. The same method was used to get the maximum yeast number for Inp54p cells without recruitment.

Western Blotting

Sample (N150-GFP-T2A-mCherry) and positive control (N150-GFP and PHcarc-mCherry co-expression) were resuspended in SDS sample buffer and boiled for 5 min. Electrophoresis was performed using 4–15% Tris·HCl polyacrylamide gels (#5671085, Criterion, Bio-Rad), and proteins were then transferred to a polyvinylidene fluoride membrane. The membranes were blocked and incubated with primary antibodies in 3% BSA in TBST overnight. Then specific fluorescent conjugated secondary antibody was applied and prepared for imaging as per manufacturer's protocol (LI-COR Biosciences). Blots were then imaged on a LI-COR Odyssey CLx at medium resolution. The primary antibodies used in the studies were: Anti-GFP antibody (mouse, monoclonal; Roche); Anti-mCherry antibody (mouse, monoclonal; Abcam).

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